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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
A61K 38/08, C07K 7/14, C12N 5/06, 5/08, 5/00	A1	WO 99/42122
		(43) International Publication Date: 26 August 1999 (26.08.99)

(21) International Application Number: PCT/US99/03243

(22) International Filing Date: 16 February 1999 (16.02.99)

(30) Priority Data:
60/075,179 19 February 1998 (19.02.98) US

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(81) Designated States: AU, CA, JP, MX, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD OF PROMOTING EMBRYONIC STEM CELL PROLIFERATION

(57) Abstract

The present invention provides methods, improved cell culture medium and kits for promoting embryonic stem cell proliferation by growth in the presence of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments or analogues thereof, or AII AT_2 type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

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METHOD OF PROMOTING EMBRYONIC STEM CELL PROLIFERATION

Cross Reference

This application is a continuation in part of U.S. Provisional Application 60/075,179 filed February 19, 1998.

Field of the Invention

This present invention relates to methods for accelerating the proliferation of embryonic stem cells.

Background of the Invention

Embryonic stem (ES) cells may be maintained as totipotent cells *in vitro* for many generations. In the mouse, such cells have been demonstrated as continuously growing cell lines derived from the inner cell mass of 3.5 day blastocysts of the mouse (Wiles and Keller, *Development* 111:259-267 (1991)). Reintroduction of such cells into the mouse blastocysts illustrates the ability of ES cells to generate all lineages of mouse cells (Bradley, et al. *Nature* 309:255-256 (1984)). Interestingly, when allowed to form three-dimensional structures known as embryoid bodies (EBs), ES cells will differentiate into many cell types including those of the hematopoietic system (Evans and Kaufman, *Nature* 292:154-156 (1981); Martin, *Proc. Natl. Acad. Sci.* 78:7634-7638 (1981); Doetschman *et al. J. Embryol. Exp. Morphol.* 87:27-45 (1985)). Thus, ES cells provides a unique system with which to

analyze both the cellular and molecular events involved in the first stages of lineage determination.

Replacement therapy, in which ES cells are administered to a patient having a deficiency in cells of a certain or multiple lineages, would be of great use to those skilled in the art. A large number of recombinant growth factors that act at different stages in the system are available (Wiles and Keller, 1991). However, these factors have not been proven to be sufficient for utilization in replacement therapy. In order to utilize ES cells for therapy of disease states requiring replacement therapy, reagents and methods which drive proliferation of ES cells must be developed. Methods that increase the *ex vivo* proliferation of embryonic stem cells will greatly increase the utility of replacement therapy. Similarly, methods that increase *in vivo* proliferation of stem cells will enhance the utility of replacement therapy by rapidly increasing local concentrations of embryonic and lineage-committed stem cells at the site of therapy.

Summary of the Invention

The present invention provides methods that increase proliferation of embryonic stem cells that are useful in rapidly providing a large population of such cells for use in replacement therapy and for making a large population of transfected embryonic stem cells for use in replacement therapy.

In one aspect, the present invention provides methods that promote embryonic stem cell proliferation by contacting the cells with angiotensinogen, angiotensin I ("AI"), AI analogues, AI fragments and analogues thereof, angiotensin II ("AII"), AII analogues, AII fragments or analogues thereof or AII AT_2 type 2

receptor agonists, either alone or in combination with other growth factors and cytokines.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation of embryonic stem cells, wherein the improvement comprises addition to the cell culture medium of an effective amount of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists.

In a further aspect, the present invention provides kits for the propagation of embryonic stem cells, wherein the kits comprise an effective amount of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists, and instructions for culturing the cells. Preferred embodiments of the kit further comprise cell culture growth medium, a sterile container, other growth factors, and an antibiotic supplement.

Detailed Description of the Preferred Embodiments

As defined herein, the term "stem cells" refers to totipotent cells having the ability to differentiate into many different cell lineages. One such cell type is the embryonic stem cell ("ES"). As defined herein, "proliferation" encompasses both cellular self renewal and cellular proliferation with accompanying differentiation.

Unless otherwise indicated, the term "active agents" as used herein refers to the group of compounds comprising angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments or analogues thereof and AII AT_2 type 2 receptor agonists.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991, Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of healing of wound tissue, comprising the application to such tissue of angiotensin II (AII) in an amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen. The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the converting enzyme angiotensinase which removes the C-terminal His-Leu residues from AI (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu [SEQ ID NO:37]). AII is a known pressor agent and is commercially available. The use of AII analogues and

fragments, AT2 agonists, as well as AIII and AIII analogues and fragments in wound healing has also been described. (U.S. Patent No. 5,629,292; U.S. Patent No. 5,716,935; WO 96/39164; all references herein incorporated by reference in their entirety.)

Studies have shown that AII increases mitogenesis and chemotaxis in cultured cells that are involved in wound repair, and also increases their release of growth factors and extracellular matrices (diZerega, U.S. Patent No. 5,015,629; Dzau et. al., *J. Mol. Cell. Cardiol.* 21:S7 (Supp III) 1989; Berk et. al., *Hypertension* 13:305-14 (1989); Kawahara, et al., *BBRC* 150:52-9 (1988); Naftilan, et al., *J. Clin. Invest.* 83:1419-23 (1989); Taubman et al., *J. Biol. Chem* 264:526-530 (1989); Nakahara, et al., *BBRC* 184:811-8 (1992); Stouffer and Owens, *Circ. Res.* 70:820 (1992); Wolf, et al., *Am. J. Pathol.* 140:95-107 (1992); Bell and Madri, *Am. J. Pathol.* 137:7-12 (1990). In addition, AII was shown to be angiogenic in rabbit corneal eye and chick chorioallantoic membrane models (Fernandez, et al., *J. Lab. Clin. Med.* 105:141 (1985); LeNoble, et al., *Eur. J. Pharmacol.* 195:305-6 (1991).

Although AII has been shown to increase the proliferation of a number of cell types *in vitro*, it does not necessarily increase the proliferation of all cell types. AII has been shown to increase cellular proliferation in hair follicles in the area of a thermal injury. (Rodgers et al., J. Burn Care Rehabil. 18:381-388 (1997). The effect of AII on a given cell type has been hypothesized to be dependent, in part, upon the AII receptor subtypes the cell expresses (Shanugam et al., *Am. J. Physiol.* 268:F922-F930 (1995); Helin et al., *Annals of Medicine* 29:23-29 (1997); Bedecs et al., *Biochem J.* 325:449-454 (1997)). These studies have shown that AII receptor subtype expression is a dynamic process that changes during development, at least in

some cell types, including the late gestation rodent fetus (Zemel et al., *Clinical Endocrinology and Metabolism* 71:1003-1007 (1990)). AII has been hypothesized to be involved in fetal development in all mammalian species (*Id.*) However, it is not known whether angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists would be useful in accelerating the proliferation of embryonic stem cells.

A peptide agonist selective for the AT2 receptor (AII has 100 times higher affinity for AT2 than AT1) is p-aminophenylalanine6-AII ["(p-NH_2-Phe)6-AII)"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein Xaa is p-NH_2-Phe (Speth and Kim, *BBRC* 169:997-1006 (1990). This peptide gave binding characteristics comparable to AT2 antagonists in the experimental models tested (Catalioto, et al., *Eur. J. Pharmacol.* 256:93-97 (1994); Bryson, et al., *Eur. J. Pharmacol.* 225:119-127 (1992).

The effects of AII receptor and AII receptor antagonists have been examined in two experimental models of vascular injury and repair which suggest that both AII receptor subtypes (AT1 and AT2) play a role in wound healing (Janiak et al., *Hypertension* 20:737-45 (1992); Prescott, et al., *Am. J. Pathol.* 139:1291-1296 (1991); Kauffman, et al., *Life Sci.* 49:223-228 (1991); Viswanathan, et al., *Peptides* 13:783-786 (1992); Kimura, et al., *BBRC* 187:1083-1090 (1992).

Many studies have focused upon AII(1-7) (AII residues 1-7) or other fragments of AII to evaluate their activity. AII(1-7) elicits some, but not the full range of effects elicited by AII. (Pfeilschifter, et al., *Eur. J. Pharmacol.* 225:57-62 (1992); Jaiswal, et al., *Hypertension* 19(Supp. II):II-49-II-55 (1992); Edwards and

Stack, *J. Pharmacol. Exper. Ther.* 266:506-510 (1993); Jaiswal, et al., *J. Pharmacol. Exper. Ther.* 265:664-673 (1991); Jaiswal, et al., *Hypertension* 17:1115-1120 (1991); Portsi, et a., *Br. J. Pharmacol.* 111:652-654 (1994).

As hereinafter defined, a preferred class of AT2 agonists for use in accordance with the present invention comprises angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, angiotensin II analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists having p-NH-Phe in a position corresponding to a position 6 of AII. In addition to peptide agents, various nonpeptidic agents (e.g., peptidomimetics) having the requisite AT2 agonist activity are further contemplated for use in accordance with the present invention.

The active angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, angiotensin II analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists of particular interest in accordance with the present invention are characterized as comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

Compounds falling within the category of AT2 agonists useful in the practice of the invention include the AII analogues set forth above subject to the restriction that R^6 is p-NH_2-Phe.

Particularly preferred combinations for R^A and R^B are Asp-Arg, Asp-Lys, Glu-Arg and Glu-Lys. Particularly preferred embodiments of this class include the following: AII, AIII or AII(2-8), Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]; AII(3-8), also known as des1-AIII or AIV, Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:3]; AII(1-7), Asp-Arg-Val-Tyr-Ile-His-Pro {SEQ ID NO:4]; AII(2-7). Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:5]; AII(3-7), Val-Tyr-Ile-His-Pro [SEQ ID NO:6]; AII(5-8), Ile-His-Pro-Phe [SEQ ID NO:7]; AII(1-6), Asp-Arg-Val-Tyr-Ile-His [SEQ ID NO:8]; AII(1-5), Asp-Arg-Val-Tyr-Ile [SEQ ID NO:9]; AII(1-4), Asp-Arg-Val-Tyr [SEQ ID NO:10]; and AII(1-3), Asp-Arg-Val [SEQ ID NO:11]. Other preferred embodiments include: Arg-norLeu-Tyr-Ile-His-Pro-Phe [SEQ ID NO:12] and Arg-Val-Tyr-norLeu-His-Pro-Phe [SEQ ID NO:13]. Still another preferred embodiment

encompassed within the scope of the invention is a peptide having the sequence Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe [SEQ ID NO:31]. AII(6-8), His-Pro-Phe [SEQ ID NO:14] and AII(4-8), Tyr-Ile-His-Pro-Phe [SEQ ID NO:15] were also tested and found not to be effective.

A further class of particularly preferred compounds in accordance with the present invention consists of those with the following general structure:

R1-ARG-R2-TYR-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and aminoPhe; and

R5 is either Phe or H.

Preferred embodiments of this class of the invention include SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:38. Particularly preferred embodiments of this class include SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:18, and SEQ ID NO:38.

Another class of compounds of particular interest in accordance with the present invention are those of the general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

A particularly preferred subclass of the compounds of general formula II has the formula

R^2-R^3-Tyr-R^5-His-Pro-Phe [SEQ ID NO:16]

wherein R^2, R^3 and R^5 are as previously defined. Particularly preferred is angiotensin III of the formula Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]. Other preferred compounds include peptides having the structures Arg-Val-Tyr-Gly-His-Pro-Phe [SEQ ID NO:17] and Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:18]. The fragment AII(4-8) was ineffective in repeated tests; this is believed to be due to the exposed tyrosine on the N-terminus.

In the above formulas, the standard three-letter abbreviations for amino acid residues are employed. In the absence of an indication to the contrary, the L-form of the amino acid is intended. Other residues are abbreviated as follows:

TABLE 1

Abbreviation for Amino Acids

Me^2Gly	N,N-dimethylglycyl
Bet	1-carboxy-N,N,N-trimethylmethanaminium hydroxide inner salt (betaine)
Suc	Succinyl
Phe(Br)	p-bromo-L-phenylalanyl
AzaTyr	Aza-α'-homo-L-tyrosyl
Acpc	1-aminocyclopentane carboxylic acid
Aib	2-aminoisobutyric acid
Sar	N-methylglycyl (sarcosine)

It has been suggested that AII and its analogues adopt either a *gamma* or a *beta* turn (Regoli, et al., Pharmacological Reviews 26:69 (1974). In general, it is believed that neutral side chains in position R^3, R^5 and R^7 may be involved in maintaining the appropriate distance between active groups in positions R^4, R^6 and R^8 primarily responsible for binding to receptors and/or intrinsic activity. Hydrophobic side chains in positions R^3, R^5 and R^8 may also play an important role in the whole conformation of the peptide and/or contribute to the formation of a hypothetical hydrophobic pocket.

Appropriate side chains on the amino acid in position R^2 may contribute to affinity of the compounds for target receptors and/or play an important role in the conformation of the peptide. For this reason, Arg and Lys are particularly preferred as R^2.

For purposes of the present invention, it is believed that R^3 may be involved in the formation of linear or nonlinear hydrogen bonds with R^5 (in the gamma turn model) or R^6 (in the beta turn model). R^3 would also participate in the first turn in a beta antiparallel structure (which has also been proposed as a possible structure). In contrast to other positions in general formula I, it appears that beta and gamma branching are equally effective in this position. Moreover, a single hydrogen bond may be sufficient to maintain a relatively stable conformation. Accordingly, R^3 may suitably be selected from Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr. In another preferred embodiment, R^3 is Lys.

With respect to R^4, conformational analyses have suggested that the side chain in this position (as well as in R^3 and R^5) contribute to a hydrophobic cluster believed to be essential for occupation and stimulation of receptors. Thus, R^4 is preferably selected from Tyr, Thr, Tyr $(PO_3)_2$, homoSer, Ser and azaTyr. In this position, Tyr is particularly preferred as it may form a hydrogen bond with the receptor site capable of accepting a hydrogen from the phenolic hydroxyl (Regoli, et al. (1974), *supra*). In a further preferred embodiment, R^4 is Ala.

In position R^5, an amino acid with a β aliphatic or alicyclic chain is particularly desirable. Therefore, while Gly is suitable in position R^5, it is preferred that the amino acid in this position be selected from Ile, Ala, Leu, norLeu, Gly and Val.

In the AII analogues, fragments and analogues of fragments of particular interest in accordance with the present invention, R^6 is His, Arg or 6-NH_2-Phe. The unique properties of the imidazole ring of histidine (e.g., ionization at physiological pH, ability to act as proton donor or acceptor, aromatic character) are believed to

contribute to its particular utility as R^6. For example, conformational models suggest that His may participate in hydrogen bond formation (in the *beta* model) or in the second turn of the antiparallel structure by influencing the orientation of R^7. Similarly, it is presently considered that R^7 should be Pro in order to provide the most desirable orientation of R^8. In position R^8, both a hydrophobic ring and an anionic carboxyl terminal appear to be particularly useful in binding of the analogues of interest to receptors; therefore, Tyr and especially Phe are preferred for purposes of the present invention.

Analogues of particular interest include the following:

TABLE 2: Angiotensin II Analogues

AII Analogue Name	Amino Acid Sequence	Sequence Identifier
Analogue 1	Asp-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 19
Analogue 2	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 20
Analogue 3	Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe	SEQ ID NO: 21
Analogue 4	Glu-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 22
Analogue 5	Asp-Lys-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 23
Analogue 6	Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 24
Analogue 7	Asp-Arg-Val-Thr-Ile-His-Pro-Phe	SEQ ID NO: 25
Analogue 8	Asp-Arg-Val-Tyr-Leu-His-Pro-Phe	SEQ ID NO: 26
Analogue 9	Asp-Arg-Val-Tyr-Ile-Arg-Pro-Phe	SEQ ID NO: 27
Analogue 10	Asp-Arg-Val-Tyr-Ile-His-Ala-Phe	SEQ ID NO: 28
Analogue 11	Asp-Arg-Val-Tyr-Ile-His-Pro-Tyr	SEQ ID NO: 29
Analogue 12	Pro-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 30
Analogue 13	Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 31
Analogue 14	Asp-Arg-Val-Tyr$(PO_3)_2$-Ile-His-Pro-Phe	SEQ ID NO: 32
Analogue 15	Asp-Arg-norLeu-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 33
Analogue 16	Asp-Arg-Val-Tyr-norLeu-His-Pro-Phe	SEQ ID NO: 34
Analogue 17	Asp-Arg-Val-homoSer-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 35

The polypeptides of the instant invention may be synthesized by methods such as those set forth in J. M. Stewart and J. D. Young, *Solid Phase Peptide*

Synthesis, 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984) and J. Meienhofer, *Hormonal Proteins and Peptides*, Vol. 2, Academic Press, New York, (1973) for solid phase synthesis and E. Schroder and K. Lubke, *The Peptides*, Vol. 1, Academic Press, New York, (1965) for solution synthesis. The disclosures of the foregoing treatises are incorporated by reference herein.

In general, these methods involve the sequential addition of protected amino acids to a growing peptide chain (U.S. Patent No. 5,693,616, herein incorporated by reference in its entirety). Normally, either the amino or carboxyl group of the first amino acid and any reactive side chain group are protected. This protected amino acid is then either attached to an inert solid support, or utilized in solution, and the next amino acid in the sequence, also suitably protected, is added under conditions amenable to formation of the amide linkage. After all the desired amino acids have been linked in the proper sequence, protecting groups and any solid support are removed to afford the crude polypeptide. The polypeptide is desalted and purified, preferably chromatographically, to yield the final product.

In one aspect of the present invention, a method of increasing *in vitro* and *ex vivo* embryonic stem cell proliferation by exposure to angiotensinogen, AI, AI analogues, AI fragments, AII, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists ("active agents") is disclosed. Experimental conditions for the isolation, purification, *ex vivo* growth and *in vivo* mobilization of embryonic stem cells have been reported (Wulf, et al. 1993. *EMBO*, 12(13): 5065-5074; Wiles, 1991. *Development*, 111:259-267; Guillemot, et al. 1996. *Blood*, 88(7): 2722-2731). Embryonic stem cells may be obtained freshly from a host such as a murine animal, e.g. a mouse, rat, guinea pig, chinese hamster or other small

laboratory animals. The cells may be grown on an appropriate fibroblast-feeder layer or grown in the presence of leukemia inhibiting factor (LIF). (U.S. Patent No. 5,574,205, incorporated by reference herein in its entirety).

In a preferred embodiment, a G418-resistant subclone (CCEG2) of the 129/Sv-derived ES line CCE (Robertson *et al.*, 1986. *Nature*, 323: 445-448), adapted to grow in the presence of LIF and without feeder cells, is used. To maintain the cells in an undifferentiated state, they are subcultured regularly onto gelatin (0.1% swine skin-300 bloom) treated tissue culture flasks in Dulbecco's modified Eagle's medium (DME) supplemented with 15% fetal calf serum (FCS; selected batch), 1000 units/ml leukemia inhibitory factor (LIF; Genetics Institute, Cambridge, MA) and 1.5×10^{-4} M (12.6 μl) monothioglycerol (MTG). Under these conditions, more than 95% of the cell population remains undifferentiated as determined by visual inspection under phase-contrast microscopy. The ES cell line was derived from D3 which was originally derived from a 129/Sv mouse (Doetschman *et al.*, 1985. *J. Embryol. Exp. Morph.*, 87: 27-45).

Embryonic stem cells are suspended in culture medium and incubated in the presence of, preferably, between about 0.1 ng/ml and about 10 mg/ml of the active agents of the invention. The cells are expanded for a period of between 8 and 21 days and cellular proliferation is assessed via any one of a variety of techniques well known in the art, including, but not limited to, bromodeoxyuridine incorporation (Vicario-Abejon et al., 1995), ^{3}H-thymidine incorporation (Fredericksen et al., 1988), or antibody labeling of a protein present in higher concentration in proliferating cells than in non-proliferating cells. In a preferred embodiment, proliferation of embryonic stem cells is assessed by reactivity to an antibody

directed against a protein known to be present in higher concentrations in proliferating cells than in non-proliferating cells, including but not limited to proliferating cell nuclear antigen (PCNA, or cyclin; Zymed Laboratories, South San Francisco, California). Viable cells may also be identified using a technique such as the trypan blue exclusion assay.

In a further preferred embodiment, stem cells that have been cultured in the presence of the active agents are used, either alone or in combination with another compound of interest such as a cytokine, for studying embryonic stem cell proliferation in the research laboratory. Proliferation is then measured as described above.

In addition, mechanisms of gene expression during proliferation of embryonic stem cells may be studied using the methodologies of the instant invention. The embryonic stem cells may be exposed to the active agents and the level of gene expression, either specifically or generally, is determined. This may be accomplished by any of several techniques well known and widely available to one skilled in the art. Such techniques include nuclease protection assay (Multi-NPA, Ambion, Inc., Austin, TX), northern blot, RT-PCR, competitive PCR, relative PCR, RNase protection (RPA II, Ambion, Inc., Austin, TX).

To perform the assay, the cells are rinsed to remove all traces of culture fluid, resuspended in an appropriate medium and then pelleted and rinsed several times. After the final rinse, the cells are resuspended at between 0.7×10^6 and 50×10^6 cells per ml in an appropriate medium containing an effective amount (generally 0.1 ng/ml to 10 mg/ml) of the active agents. The cells are then incubated for an appropriate period of time and assayed for proliferation as described above.

Alternatively, RNA may be isolated and an assay performed to determine the level of expression of RNA encoding a protein expressed in proliferating cells such as PCNA.

In another embodiment, assessment of the *in vivo* proliferative effect of the active agents on stem cells is done by histochemical evaluations of various tissues. In a preferred embodiment, *in vivo* proliferation of ES cells is assessed by reactivity to an antibody directed against a protein known to be present in higher concentrations in proliferating ES cells than in non-proliferating cells, such as proliferating cell nuclear antigen (PCNA, or cyclin; Zymed Laboratories). Embryonic stem cell proliferation may also be assessed by examination of changes in gene expression, phenotype, morpholgy, by genetic detection (for example, the donor cell is engineered to differ from the recipient by having a different allele of one gene), or any other method that distinguishes proliferating stem cells from non-proliferating stem cells.

In a preferred embodiment, the stem cell may be transfected with a marker gene that that allows for genetic detection of the transfected cell. Following administration of such a transfected stem cell after exposure of cells to the active agents, the stem cell is administered to a patient having a deficiency in one or more cells of the same or various lineages. At certain time intervals, a sample, which may comprise a bodily fluid such as blood or a tissue sample, is removed from the patient and the presence and quantity of stem cells in the patient may be measured by detection of the marker gene. The amount of marker gene detected indicates the amount of stem cells present in the patient at a certain point in time. The marker gene may be detected by any of several techniques well known and widely available

to one skilled in the art. Such techniques may include Southern blot, Northern blot, PCR, RT-PCR, and RNase protection assay. Alternatively, the cells can be transfected with a drug resistance marker gene, such as the neomycin resistance gene. In this case, the cells are cultured in the presence of a drug, such as neomycin, to which expression of the *neo* gene confers resistance. Only those cells that express the drug resistance gene will survive exposure to the drug, allowing for quantitation of the number of embryonic stem cells in the sample.

For use in increasing proliferation of stem cells, the active agents may be administered at a level of between about 0.1 ng/kg to about 10 mg/kg. The active agents may be administered by any suitable route, including orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intra-arterial, intravenous, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally.

The active agents may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions) and may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

While the active agents of the invention can be administered as the sole active agent, they can also be used in combination with one or more other compounds. When administered as a combination, the active agents and other compounds can be formulated as separate compositions that are given at the same

time or different times, or the active agents and other compounds can be given as a single composition. In a preferred embodiment, the active agents are administered in combination with leukemia inhibitory factor.

For administration, the active agents are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The active agents may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The dosage regimen for increasing *in vivo* proliferation of stem cells with the active agents is based on a variety of factors, including the type of injury or deficiency, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed.

Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Dosage levels of the order of between 0.1 ng/kg and 10 mg/kg of the active agents are useful for all methods of use disclosed herein.

In a preferred embodiment of the present invention, the active agents are administered topically. A suitable topical dose of active ingredient of the active agents is preferably between about 0.1 mg/kg and about 10 mg/kg administered twice daily. For topical administration, the active ingredient may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation of stem cells, wherein the improvement comprises addition to the cell culture medium of an effective amount of the active agents of the invention, as described above. Any cell culture media that can support the growth of stem cells can be used with the present invention. Such cell culture media include, but are not limited to Basal Media Eagle, Dulbecco's Modified Eagle Medium, Iscove's Modified Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, F-10 Nutrient Mixtures, Opti-MEM® Reduced-Serum Medium, RPMI Medium, and Macrophage-SFM Medium or combinations thereof.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as either a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement. Culture media is commercially available from many sources, such as GIBCO BRL (Gaithersburg, MD) and Sigma (St. Louis, MO)

In a further aspect, the present invention provides kits for the propagation of embryonic stem cells, wherein the kits comprise an effective amount of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists, as described above.

In a preferred embodiment, the kit further comprises cell culture growth medium. Any cell culture media that can support the growth of embryonic stem cells can be used with the present invention. Examples of such cell culture media are described above.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as either a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement.

In another preferred embodiment, the kit further comprises a sterile container. The sterile container can comprise either a sealed container, such as a cell culture flask, a roller bottle, or a centrifuge tube, or a non-sealed container, such as a cell culture plate or microtiter plate (Nunc; Naperville, IL).

In a further preferred embodiment, the kit further comprises an amount of leukemia inhibitory factor (LIF) sufficient to prevent differentiation to cells beyond the ESC. In a most preferred embodiment, the kit comprises between about 10 units/ml and about 1000 units/ml of LIF.

In a further preferred embodiment, the kit further comprises an antibiotic supplement for inclusion in the reconstituted cell growth medium. Examples of appropriate antibiotic supplements include, but are not limited to actimonycin D,

Fungizone®, kanamycin, neomycin, nystatin, penicillin, streptomycin, or combinations thereof (GIBCO).

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Example 1. AII Stimulation of Embryonal Cell Proliferation

Preparation of Embryonic Fibroblast (EF) Cells

EF cells are feeder cells used to produce proper culture conditions for the proliferation of the embryonal stem cells (ESC). EF cells were isolated from mouse embryos at day 14 of gestation (the kind gift of Dr. Peter Laird, Univ. of Southern California) by the following procedure:

1) Embryos were isolated and washed once in HEPES buffered saline;
2) The individual embryo was dissected to remove the head and soft tissues and the carcass was washed twice with HEPES-buffered saline;
3) The embryo carcasses were minced into fine pieces in a small volume of trypsin/EDTA solution (2 ml for 10 embryos);
4) The tissue was mixed well and incubated for 30 minutes at 37°C;
5) Ten ml of DMEM with 10% FCS was added and the digested tissue was transferred to a 50 ml tube and the tissues were disassociated with vigorous pipeting;
6) The large pieces of tissue debris were allowed to settle and the supernatant was transferred to a clean tube.
7) Steps 5 and 6 were repeated four additional times with the tissue pieces;
8) All of the supernatants were combined and the cells suspension was plated out into 175 cm^2 flasks (approximately one embryo per flask);
9) After 24 hours, the feeder cells were frozen for future expansion

Expansion and Mitotic Inactivation of EF Cells

A tube of primary EF cells (described above) was thawed and expanded for several passages in HEPES-buffered (pH 7.3) DMEM, high glucose, supplemented with 10% fetal calf serum (FCS) and antibiotics. When sufficient numbers of cells were isolated (confluence in 12 175-cm^2 flasks), the cells were subjected to 2000-3000 rads of irradiation to inactivate proliferation. After irradiation, the EF cells were frozen for several months in liquid nitrogen before use. Cells from each flask were trypsinized and resuspended in 12.5 ml of DMEM with 10% FCS followed by addition of 12.5 ml of 2X freezing medium (20% DMSO, 20% FCS in HEPES-buffered DMEM). These cells are frozen in 1 ml aliquots which contains feeder cells for 200 cm^2.

Preparation of Feeder Cells (EF Cells)

Twenty five cm^2 flasks were coated with 0.2% gelatin (Stem Cell Technologies, Vancouver, BC) for 15 minutes at room temperature. After removal of extraneous gelatin, the feeder cells were thawed and placed in the flasks (one vial of irradiated feeders was sufficient for 8 25 cm^2 flasks). After preparation of the feeder layer for 24 hours, the mouse embryonal cells, isolated via standard procedures (Martin, Proc. Natl. Acad. Sci. 78:7634-7638 (1981); Evans and Kaufman, Nature 292:154-156 (1981), were thawed and added to the feeder layer. These cultures were fed daily with 5 ml of DMEM containing 15% FCS, 0.1 mM non-essential amino acids, antibiotics (penicillin and streptomycin), 0.1 mM 2-mercaptoethanol and 500 units/ml Leukemia Inhibitory Factor (LIF) (Stem Cell Technologies, Vancouver, BC).

Evaluation of the Effects of AII on the Proliferation of ESC

After growing to healthy colonies, but prior to differentiation (approximately 3 days after thawing), cells were harvested for assessment of the effect of AII on the proliferation of ESC. The ESC were trypsinized from the tissue culture flask together with the feeder cells for replating under the appropriate conditions. Flasks containing ESC to be passaged were washed with 3-4 ml of HEPES-buffered saline, and 0.5 ml of 0.25% trypsin/1 mM EDTA was added. The cells were incubated at 37°C for 2-3 minutes. The flask was then shaken to detach the cells and 2 ml of ES medium was added. This mixture was pipetted up and down to achieve a single cell suspension. In the initial study, 5 x 10^3 cells were added per well in 96 well plates in various media with and without feeder cells. The proliferation of the cells in ES medium with and without LIF was determined. After 24 hours, various concentrations of AII were added to the wells. Three days after addition of AII, the number of cells per well was assessed (Table 1). The data show that AII was most effective at accelerating the proliferation of ES cells in the presence of LIF.

Table 1. Effect of AII in the Medium of the Proliferation of ES Cells

Concentration of AII (μg/ml)

Medium	0	1	10	100
EF w/o LIF	8 x 10^4 cells/ml	8 x 10^4 cells/ml	7 x 10^4 cells/ml	7x10^4 cells/ml
EF with LIF	8 x 10^4 cells/ml	11 x 10^4 cells/ml	15 x 10^4 cells/ml	15 x 10^4 cells/ml
No EF or LIF	7 x 10^4 cells/ml	7 x 10^4 cells/ml	9 x 10^4 cells/ml	9 x 10^4 cells/ml
LIF only	5 x 10^4 cells/ml	14 x 10^4 cells/ml	11 x 10^4 cells/ml	12 x 10^4 cells/ml

In a further experiment, the ESC were trypsinized from the tissue culture flask together with the feeder cells for replating under appropriate conditions. The

flask containing ESC to be passaged was washed with 3-4 ml of HEPES-buffered saline, and 0.5 ml of 0.25% trypsin/1 mM EDTA was added. The cells were incubated at 37°C for 2-3 minutes. The flask was then shaken to detach the cells and 2 ml of ES medium was added. This mixture was pipetted up and down to achieve a single cell suspension. In the initial study, 1 x 10^3 cells were added per well in 96 well plates in various media with and without feeder cells. The formation of colonies of ES cells in ES medium with and without LIF was determined. After 24 hours, various concentrations of AII were added to the wells. Three days after addition of AII, the number of colonies per well was assessed (Table 1). In the absence of feeder cells, the ESC did not adhere to the plate and colonies did not form. While the number of colonies that formed was increased by AII both in the presence and absence of LIF, the increase was much greater in the presence of LIF (Table 2).

Table 2. The Effect of AII in the Culture Medium on the Formation of Colonies of ESC

Concentration of AII (μg/ml)

Medium	0	1	10	100
Without LIF	670 Colonies	860 Colonies	960 Colonies	940 Colonies
With LIF	480 Colonies	1200 Colonies	1288 Colonies	1345 Colonies

The present invention, by providing a method for enhanced proliferation of embryonic stem cells, will greatly increase the clinical benefits of embryonic stem transplantation. This is true both for increased "self-renewal", which will provide a larger supply of embryonic stem cells capable of generating a lineage of cells that are deficient in the patient, and for proliferation with differentiation which will provide a larger supply of the progenitor cells of different cell lineages. Similarly,

methods that increase *in vivo* proliferation of embryonic stem cells will enhance the utility of replacement therapy by rapidly increasing local concentrations of the stem cells and their progeny at the site of therapy.

Example 2. Effect of Various Peptides on the Proliferation of Embryonal Stem Cells in the Presence of LIF

These studies were preformed as in Example 1, in the absence of feeder cells. The number of cells per well was evaluated in the presence and absence of LIF. The data were gathered 4 days after initiation of the cultures,

Table 3. Effect of Various Peptides on the Proliferation of Embryonal Stem Cells in the Presence of LIF

Concentration of Peptide in Culture

Peptide	0 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml
Control	0.9×10^5			
AII		1.8×10^5	2.4×10^5	2.2×10^5
Ala3aminoPhe6AII		2.0×10^5	2.0×10^5	2.0×10^5
Ala4-AII		1.0×10^5	1.4×10^5	1.7×10^5
AII(1-7)		2.0×10^5	4.4×10^5	3.0×10^5

In the table, the peptides are identified as follows:

AII:	SEQ ID NO:2
Ala3aminoPhe6 AII:	SEQ IDNO:38
Ala4-AII	SEQ ID NO:18
AII(1-7)	SEQ ID NO:4

Table 4. Effect of Various Peptides on the Proliferation of Embryonal Stem Cells in the Absence of LIF

Concentration of Peptide in Culture

Peptide	0 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml
Control	0.8×10^5			
AII		0.5×10^5	1.2×10^5	1.0×10^5
Ala3aminoPhe6AII		1.0×10^5	0.8×10^5	0.6×10^5
Ala3-AIII		0.6×10^5	0.8×10^5	0.8×10^5
AII(1-7)		0.5×10^5	0.6×10^5	0.6×10^5

These data demonstrate that the various peptides all stimulated proliferation of ESC in the presence of LIF.

The method of the present invention also increases the potential utility of embryonic stem cells as vehicles for gene therapy in certain disorders by more efficiently providing a large number of such cells for transfection, and also by providing a more efficient means to rapidly expand transfected embryonic stem cells.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.

We claim:

1. A method for promoting embryonal cell proliferation comprising contacting embryonal cells with an amount effective to promote proliferation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys, and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

2. The method of claim 1 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO. 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO: 37; and SEQ ID NO:38.

3. The method of claim 1 wherein the active agent is SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:38.

4. The method of claim 1 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

5. An improved cell culture medium for promotion of embryonal cell proliferation, wherein the improvement comprises addition to the cell culture medium an amount effective to increase proliferation of embryonal cells of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, $Glu(NH_2)$, Gly, $Asp(NH_2)$ and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys, and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

6. The improved cell culture medium of claim 5 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO. 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO: 37; and SEQ ID NO:38.

7. The improved cell culture of claim 5 wherein the active agent is SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:38.

8. The improved cell culture medium of claim 5 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

9. A kit for promoting embryonal cell proliferation comprising:

(a) an amount effective to promote embryonal cell proliferation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys, and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group; and

(b) instructions for using the amount effective of active agent to promote embryonal cell proliferation.

10. The kit of claim 9 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO. 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO: 37; and SEQ ID NO:38.

11. The kit of claim 9 wherein the active agent is SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:38.

12. The kit of claim 9 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

13. A pharmaceutical composition comprising an amount effective to promote embryonal cell proliferation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, $Glu(NH_2)$, Gly, $Asp(NH_2)$ and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys, and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

an amount of leukemia inhibitory factor effective to prevent differentiation of embryonal stem cells; and

a pharmaceutically acceptable carrier.

14. The pharmaceutical composition of claim 13 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO. 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29,

SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO: 37; and SEQ ID NO:38.

15. The pharmaceutical composition of claim 13 wherein the active agent is SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:38..

16. The pharmaceutical composition of claim 13 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

17. A method for promoting embryonal cell proliferation comprising contacting embryonal cells with an amount effective to promote proliferation of at least one active agent comprising a sequence consisting of the general formula I

R1-ARG-R2-TYR-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and aminoPhe; and

R5 is either Phe or H.

18. The method of claim 17 wherein the active agent is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:38.

19. The method of claim 17 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

20. An improved cell culture medium for promotion of embryonal cell proliferation, wherein the improvement comprises addition to the cell culture

medium an amount effective to increase proliferation of embryonal cells of at least one active agent comprising a sequence consisting of the general formula I

R1-ARG-R2-TYR-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and aminoPhe; and

R5 is either Phe or H.

21. The improved cell culture medium of claim 20 wherein the active agent is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:38.

22. The improved cell culture medium of claim 20 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

23. A kit for promoting embryonal cell proliferation comprising:

(a) an amount effective to promote embryonal cell proliferation of at least one active agent comprising a sequence consisting of the general formula I

R1-ARG-R2-TYR-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and aminoPhe; and

R5 is either Phe or H; and

(b) instructions for using the amount effective of active agent to promote embryonal cell proliferation.

24. The kit of claim 23 wherein the active agent is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:38.

25. The kit of claim 23 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

26. A pharmaceutical composition comprising an amount effective to promote embryonal cell proliferation of at least one active agent comprising a sequence consisting of the general formula I

R1-ARG-R2-TYR-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of H or Asp;

R2 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and aminoPhe; and

R5 is either Phe or H;

an amount of leukemia inhibitory factor effective to prevent differentiation of embryonal stem cells; and

a pharmaceutically acceptable carrier.

27. The pharmaceutical composition of claim 26 wherein the active agent is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:38.

28. The pharmaceutical composition of claim 26 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

SEQUENCE LISTING

<110> Kathleen Rodgers and Gere diZerega

<120> Method of Promoting Embryonic Stem Cell Proliferaton

<130> 98,010-B

<140> To be assigned

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Orn, Ser(Acetylated), MeGly, D-Arg, or D-Lys; Xaa at position 2 can be Val, Ala, Leu, Nle, Ile, Gly, Pro, Aib, Acp, or Tyr; Xaa at position 4 can be Ile, Ala, Leu, Nle, Val, or Gly

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1 5

INTERNATIONAL SEARCH REPORT

Int Jonal Application No
PCT/US 99/03243

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/08 C07K7/14 C12N5/06 C12N5/08 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MRUG M ET AL: "Angiotensin II stimulates proliferation of normal early erythroid progenitors" JOURNAL OF CLINICAL INVESTIGATION, XP002077069 see the whole document ---	1-4, 17-19
A,P	WO 98 32457 A (DIZEREGA GERE ;RODGERS KATHLEEN E (US); UNIV SOUTHERN CALIFORNIA () 30 July 1998 ---	
A	US 5 015 629 A (DIZEREGA GERE S) 14 May 1991 ---	
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 June 1999	09/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

1

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INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/US 99/03243

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HEATH J K ET AL: "REGULATORY FACTORS OF EMBRYONIC STEM CELLS" JOURNAL OF CELL SCIENCE, no. SUPPL. 10, 1 January 1988, pages 257-266, XP000569976	
A	WO 90 08188 A (AMRAD CORP LTD) 26 July 1990	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

.rnational application No.
PCT/US 99/03243

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1,5,9,13
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION SHEET PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99 03243

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 1,5,9,13

The scope of claim 1, 5, 9 and 13 is unduly broad and speculative. A formula consisting virtually of variables cannot be considered to be a clear and concise definition of patentable subject-matter. (Art. 6 PCT). Furthermore, the available experimental data actually only comprise a very small part of the compounds claimed, therefore the claims are also not adequately supported by the description.
Therefore, a meaningful and economically feasible search could not encompass the complete subject-matter of the claims. Consequently, the search has been limited to angiotensin I, II and (closely) related analogues, that is those encompassed by claims 17 and 18. (Art. 17(2)(a)(ii) PCT).

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 99/03243

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9832457 A	30-07-1998	AU 6648598 A	18-08-1998
US 5015629 A	14-05-1991	NONE	
WO 9008188 A	26-07-1990	AT 142695 T	15-09-1996
		AU 622515 B	09-04-1992
		AU 4835690 A	13-08-1990
		CA 2045126 A	11-07-1990
		DE 69028514 D	17-10-1996
		EP 0453453 A	30-10-1991
		JP 4502554 T	14-05-1992
		US 5641676 A	24-06-1997
		US 5418159 A	23-05-1995